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(54) Title: POLYNUCLEOTIDE VACCINE AGAINST CANINE DISTEMPER

(57) Abstract

Disclosed are polynucleotide vaccines against the canine distemper virus, methods of preparation of the polynucleotides and the vaccines, and the use of the polynucleotides and the vaccines for prophylactic immunization of mammals susceptible to canine distemper.

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## POLYNUCLEOTIDE VACCINE AGAINST CANINE DISTEMPER

### Technical Field

5 The invention concerns polynucleotide vaccines against the canine distemper virus (CDV), methods of preparation of the polynucleotides and the vaccines comprising them, and the use of the polynucleotides as  
10 vaccines for prophylactic immunization of animals susceptible to canine distemper.

### Background Art

15 Canine distemper is a highly infectious, acute or subacute, febrile viral disease of dogs and other carnivores, which occurs world-wide. Some dogs show primarily respiratory signs, others intestinal signs and at least 30% of the animals develop neurological symptoms. All experimentally infected dogs have histopathological lesions in the central nervous system. The  
20 mortality rate ranges between 30 and 80%. In a minority of cases, dogs that have recovered continue to harbour the virus in brain cells where it replicates slowly and eventually produces old dog encephalitis. The situation  
25 is analogous to that of subacute sclerosing panencephalitis in the corresponding human infection, measles. Dogs surviving distemper have life-long immunity to re-infection. Immunization is recommended for the control of  
30 distemper in dogs, using attenuated live virus vaccines at the age of 8 weeks and again at 12 to 16 weeks. Annual re-vaccination is recommended.

The importance of effective vaccines against morbillivirus infections is emphasized by recent reports  
35 on the discovery of new members of this virus group, affecting both terrestrial and marine mammals (Kennedy et al. 1988; Domingo et al. 1991). There have been several

outbreaks of canine distemper among lions of the Serengeti and lions, tigers and leopards in American zoos (Appel et al. 1994; Leary, 1994). It was surprising, that these big cats are susceptible to CDV. Furthermore, in Australia a disease of horses, acute equine respiratory syndrome (AERS) occurred and it was shown, that the AERS virus belongs to the genus morbillivirus of the paramyxoviridae (Murray, 1994). This virus not only infects horses but is also transmissible to man. Morbilliviruses thus seem to have expanded their host range. Increasing incidence of canine distemper has also been noted in Japan, Finland, Italy and Switzerland despite vaccination. The tested virus isolates were different from vaccine strains, in terms of reactivity with antibodies raised against the vaccine strains (Mori et al. 1994). In Germany and Switzerland CDV infections among wild carnivores have been reported, and mustelids may be a hidden reservoir of CDV (Alldinger et al. 1994). Recent experiments demonstrated CDV-RNA in bone tissues of humans with a chronic bone illness characterized by excessive bone resorption, new bone formation and deformity, the so-called Paget's disease (Gordon et al. 1992). Therefore, CDV has been suggested to be involved in the pathogenesis of Paget's disease. It is well known that CDV can infect bone cells of its natural host (Gordon et al. 1992; Mee et al. 1992). Moreover, bone lesions were observed in young dogs with experimental and spontaneous distemper (Baumgärtner et al. 1995). In addition to acute infections, two members of the morbilliviruses, measles virus and canine distemper virus, also produce a persistent infection.

Canine distemper is caused by CDV, a member of the genus morbillivirus (family paramyxoviridae). CDV is closely related to the viruses of measles and rinderpest.

The canine distemper virions (Fig. 1) are enveloped and contain a negative-strand RNA genome of

15'616 nucleotides which has been entirely sequenced for the cell culture adapted Onderstepoort (OP-CDV) strain (Sidhu et al., 1993, and references therein). The viral genome encodes 6 proteins: the nucleocapsid (N) protein, the phosphoprotein (P), the matrix (M) protein, the fusion (F) protein, the hemagglutinin (H) protein, and the large (L) protein. The genes are arranged in the genomic RNA in the order (3'-5'): N, P, M, F, H, and L and each protein is translated from a unique mRNA transcribed from the negative strand RNA template.

The currently used vaccines against canine distemper have a number of drawbacks. They may induce immunosuppression (M. Vandeveld, University of Berne, pers. comm.) or neurological disorders (cited in Hamburger et al., 1991). Even cases of vaccine-induced distemper have been reported (C. Green, University of Georgia; R. Higgins, University of Davis; R. Maes, University of Michigan, pers. comm.). Furthermore, these vaccines are not particularly satisfactory in terms of efficacy since cases of canine distemper in vaccinated dogs are not rare. Thus, of 84 dogs with diagnosed neurologic distemper, 32 had complete, and 21 partial vaccine coverage (Tipold et al., 1994). The incomplete protection provided by the vaccine strains is most likely the consequence of changes occurring in the virus upon cell culture adaptation. Such changes are demonstrated by the fact that after adaptation to cell lines the virus quickly loses its ability to cause disease (Bittle et al., 1962) and that loss of virulence is associated with structural alterations in the viral nucleocapsid protein (Hamburger et al., 1991). Similarly, the observation that radiolabelled hybridization probes derived from tissue culture-adapted virus failed to detect viral nucleic acids in the brain of animals infected with virulent virus is an indication that the vaccine and virulent strains differ markedly (Mitchell et al., 1987). In view of these differences it is not surprising that immunity induced by vaccine

strains is not able to provide complete protection against virulent virus.

Since the first report of protection of mice against challenge with influenza virus following intramuscular injection of DNA (Ulmer et al., 1993) it has been recognized that injection of naked nucleic acids encoding vaccine antigens represents a potent novel avenue in vaccine development (review: Montgomery et al., 1994). The advantages of nucleic acid vaccines are obvious. Such vaccines should be safe, since no live organisms are used. Furthermore, plasmid DNA is easy and cheap to produce and is stable even in adverse climatic conditions which makes DNA vaccines particularly attractive for developing countries. An additional advantage is that new plasmids can be constructed and tested in a relatively short time which is important for designing vaccines against pathogens for which the protective antigens have not yet been identified. Perhaps the most attractive feature of nucleic acid vaccines is that they induce both antibody and cell-mediated immune responses (Ulmer et al., 1993).

Several methods for delivering DNA are currently available (review: Montgomery et al., 1994). The most convenient method is direct injection into muscle tissue (Wolff et al., 1992).

#### Disclosure of the invention

Object of the presented invention is to produce novel nucleic acid vaccines against canine distemper which lack the drawbacks of hitherto vaccines against this disease. In particular, said vaccine is a polynucleotide vaccine containing virulent canine distemper virus genes which are important for eliciting neutralizing antibodies, and which are essential for cell-mediated immunity. These genes are to be inserted into expression plasmids which after delivery to living tissues produce

an immunizing effect. It is believed that a nucleic acid vaccine containing genes of virulent distemper virus has significant advantages in terms of efficacy over conventional attenuated vaccine strains which differ markedly from virulent virus. Furthermore, no reversion to virulence, which has been demonstrated for distemper virus vaccine strains (Appel, 1978) and which may result in distemper outbreaks in vaccinated animals is possible (Bush et al., 1976; Carpenter et al., 1976; Hartley et al., 1974). In addition, the inclusion of different genes in combination in the nucleic acid vaccine will generate both a humoral and a cellular immune response. A further advantage of a nucleic acid vaccine against canine distemper is that such a vaccine, in contrast to conventional live vaccine strains, will not induce immunosuppression. This is particularly important when the canine distemper vaccine is administered together with other components in a multivalent vaccine. In this situation, immunosuppression of the host renders other live vaccine components more virulent, possibly resulting in disease induced by these vaccine strains. Immunosuppression by canine distemper vaccine strains also reduces the immune response to inactivated components contained in a multivalent vaccine. A nucleic acid vaccine against canine distemper will not have these undesirable side effects. Thus, the inventive vaccine is in many aspects superior to hitherto known vaccines.

**Brief Description of the Sequence Listings  
and the Figures:**

SEQU ID NO 1 shows the primer sequence corresponding to the leader of CDV strain A75/17;

SEQU ID NO 2 shows the primer sequence corresponding to the end of the N gene of CDV strain A75/17;

SEQU ID NO 3 shows the primer sequence corresponding to the M gene at position M 116 of strain OP-CDV;

5 SEQU ID NO 4 shows the primer sequence corresponding to the F gene at position F 1092 of strain OP-CDV;

SEQU ID NO 5 shows the primer sequence corresponding to the F gene at position F 177 of strain OP-CDV;

10 SEQU ID NO 6 shows the primer sequence corresponding to the F gene at position F 2058 of strain OP-CDV;

SEQU ID NO 7 shows the primer sequence corresponding to the F gene at position F 2002 of strain OP-CDV;

15 SEQU ID NO 8 shows the primer sequence corresponding to the H gene at position H 716 of strain OP-CDV;

SEQU ID NO 9 shows the primer sequence corresponding to the H gene at position H 675 of strain OP-CDV;

20 SEQU ID NO 10 shows the primer sequence corresponding to the L gene at position L 78 of strain OP-CDV;

25 SEQU ID NO 11 shows the primer sequence for generating the 5' end of the N gene with a Kpn I restriction site;

SEQU ID NO 12 shows the primer sequence for generating the 3' end of the N gene with a Sal I restriction site;

30 SEQU ID NO 13 shows the primer sequence F1 corresponding to the F gene of strain OP-CDV at position 1 with a Mlu I restriction site;

SEQU ID NO 14 shows the primer sequence F2  
35 corresponding to the F gene of strain OP-CDV at position 2033;

SEQU ID NO 15 shows the primer sequence F3 corresponding to the F gene of strain OP-CDV at position 2014;

5 SEQU ID NO 16 shows the primer sequence F4 corresponding to the F gene of strain OP-CDV at position 2095 with a Sal I restriction site;

SEQU ID NO 17 shows the primer sequence H1 corresponding to the H gene of strain OP-CDV at position 18 with a Kpn I restriction site;

10 SEQU ID NO 18 shows the primer sequence H2 corresponding to the H gene of strain OP-CDV at position 705;

SEQU ID NO 19 shows the primer sequence H3 corresponding to the H gene of strain OP-CDV at position 15 684;

SEQU ID NO 20 shows the primer sequence H4 corresponding to the H gene of strain OP-CDV at position 1835 with a Sal I restriction site;

20 SEQU ID NO 21 shows the sequence corresponding to the N gene of virulent CDV strain A75/17. Position 1 corresponds to 5' end of the N mRNA. The translation initiation (ATG) and termination (TAA) codons are underlined;

25 SEQU ID NO 22 shows the sequence corresponding to the F gene of virulent CDV strain A75/17. Position 1 corresponds to 5' end of the F mRNA.

SEQU ID NO 23 shows the sequence corresponding to the H gene of virulent CDV strain A75/17. Position 1 corresponds to 5' end of the H mRNA.

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Figure 1 shows a schematic representation of the CDV particle. The location of the viral M, H, F, N, P and L proteins are indicated.

35 Figure 2 shows the expression plasmid H/CMV5 for the CDV H gene of strain A75/17.

Figure 3 shows the expression plasmid H/pCI for the CDV H gene of strain A75/17.



Figure 4 shows the expression plasmid N/CMV5 for the CDV N gene of strain A75/17.

Figure 5 shows the expression plasmid N/pCI for the CDV N gene of strain A75/17.

5 Figure 6 shows the expression plasmid F/CMV5 for the CDV F gene of strain A75/17.

Figure 7 shows the expression plasmid F/pCI for the CDV F gene of strain A75/17.

10 Figure 8 shows CTL assays of mice immunized with plasmid N/pCI or empty vector after 2nd immunization.

Figure 9 shows CTL assays of mice immunized with plasmid N/pCI or empty vector after 3rd immunization.

15 Figure 10 shows anti-N antibody titers of dogs immunized with standard vaccine or with plasmid N/pCI.

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#### Modes for Carrying out the Invention

In one embodiment the invention concerns a nucleic acid construct comprising a canine distemper virus gene, wherein said nucleic acid construct is capable  
25 of inducing the expression of an antigenic canine distemper virus gene product which induces a canine distemper virus specific immune response upon introduction of said nucleic acid construct into animal tissue *in vivo* and resultant uptake of the nucleic acid construct by the cells  
30 which express the encoded canine distemper virus gene.

The nucleic acid construct is a DNA or RNA construct, preferably a DNA construct.

The invention concerns in particular a nucleic acid construct, wherein the canine distemper virus  
35 gene encodes the nucleocapsid (N) protein, the phosphoprotein (P), the matrix (M) protein, the fusion (F)

protein, the hemagglutinin (H) protein, or the large (L) protein.

The nucleic acid construct is in particular such, wherein the canine distemper virus gene encodes the nucleocapsid (N) protein, the fusion (F) protein, or the hemagglutinin (H) protein.

Preferred DNA constructs are the plasmids H/CMV5 and H/pCI, which encode the hemagglutinin (H) protein, the plasmids F/CMV5 and F/pCI, which encode the fusion (F) protein of canine distemper virus strain A75/17, and in particular the plasmids N/CMV5 and N/pCI, which encode the nucleocapsid (N) protein, .

Nucleic acids coding for polypeptides of the wild-type strain A75/17 and expression vectors for the expression of such polypeptides *in vivo* are of particular importance because this strain induces distemper.

The present nucleic acid constructs are in particular expression plasmids comprising at least one and preferably one of the canine distemper genes operatively linked to a promotor and optionally to other sequences improving the expression of the gene, e.g. such as an enhancer, as well as an appropriate terminator sequence. Expression plasmids comprising such functional sequences necessary for expression of the gene are known in the art, and are e.g. plasmids CMV5 and pCI.

In another embodiment the invention concerns a polynucleotide vaccine comprising an effective amount of a nucleic acid construct, e.g. a DNA or RNA construct, and a physiologically acceptable carrier. Said vaccine induces neutralizing antibodies against canine distemper virus, canine distemper virus specific cytotoxic lymphocytes, or protective immune responses upon introduction thereof into animal tissue *in vivo*, wherein said animal is a mammal, a human, and in particular a dog.

In particular preferred is a polynucleotide vaccine comprising one or more of the plasmids selected from N/CMV5 or N/pCI, which encode the nucleocapsid (N)

protein, H/CMV5 or H/pCI, which encode the hemagglutinin (H) protein, or F/CMV5 or F/pCI which encode the fusion (F) protein of the virulent canine distemper virus strain A75/17, and a physiologically acceptable carrier.

5                   Physiologically acceptable vaccine carriers are known in the art and are e.g. physiologically acceptable injectable fluids, such as buffer solutions, e.g. phosphate-buffered saline (PBS) of appropriate pH, preferably of between about 7 to about 7.4, or injectable  
10 liposome preparations. The vaccine may also contain an adjuvant or a transfection facilitating agent. The vaccine comprises an effective, that is an immunizing amount of a nucleic acid construct of the present invention, or a combination of two or more constructs, e.g. in a concentration of about 0.01 to 100, preferably about 0.1 to  
15 1 mg /ml.

                  In yet another aspect of the invention one or more inventive constructs, each of which is carrying at least one of the canine distemper genes, are components  
20 of a multivalent vaccine. The components of said multivalent vaccine can be packed in admixed form or one or more components can be packed separately from other components but are administered either together, i.e. after mixing, or separately but almost simultaneously, i.e. a  
25 second administration directly after a first one.

                  In another embodiment the invention concerns a method for protecting an animal susceptible to infection by canine distemper virus which comprises immunization of said animal with a prophylactically effective  
30 amount of at least one polynucleotide construct comprising a gene of canine distemper virus optionally together or simultaneously with at least one other component as a multivalent vaccine.

                  A number of animals are known as being susceptible to canine distemper virus. Such animals are in  
35 particular mammals, such as carnivores, in particular dogs, and also humans.

In particular preferred is the method, wherein the polynucleotide is administered directly into tissue, preferably into muscle tissue, *in vivo*. The polynucleotide may be administered either in naked form in a physiologically acceptable solution, or contained in a liposome, or in a mixture with an adjuvant or a transfection facilitating agent. In particular preferred is the method of using a vaccine according to the present invention.

10 In another embodiment the invention concerns a method for using a canine distemper virus gene to induce an immune response *in vivo* which comprises:

- a) isolating the gene
  - b) linking the gene to regulatory sequences
- 15 such that the gene is operatively linked to control sequences which, when introduced into a living tissue, direct the transcription of the gene and subsequent translation of the mRNA, and

c) introducing the gene into a living tissue.

20 In particular preferred is the method, which comprises multiple introduction of the canine distemper gene for boosting the immune response.

In particular preferred is the method, wherein the canine distemper gene encodes the nucleocapsid (N) protein, the hemagglutinin (H) protein, or the fusion (F) protein of canine distemper virus strain A75/17.

25

In particular preferred is the method, wherein the canine distemper gene product for immunization is selected from the plasmids F/CMV5 or F/pCI, H/MCV5 or H/pCI, N/CMV5 or N/pCI which encode proteins of the wild type canine distemper virus strain A75/17, or a combination of those plasmids.

30

In another embodiment the invention concerns a composition of nucleic acid constructs encoding CDV genes from more than one canine distemper virus strain.

35

In another embodiment the invention concerns the use of an isolated canine distemper gene operatively

linked to one or more control sequences for the preparation of a vaccine for use in immunization against infection by CDV.

The following examples serve to further describe the invention, however, they should not be construed as a limitation thereof.

Example 1: Preparation of cDNA clones from canine distemper virus strain A75/17 (wild type) infected primary dog brain cell cultures

a) Preparation of cytoplasmic RNA

Primary dog brain cell cultures (DBCC) were prepared as described by Zurbriggen and Vandeveld, 1984. DBCC were infected 10-14 days after seeding, when confluency was reached, with the virulent canine distemper virus strain A75/17 (Zurbriggen et al., 1993).

About 40 days after infection, RNA was prepared from infected DBCC grown in 9-cm diameter cell culture petri dishes as follows: The medium was removed and replaced by 1ml of ice-cold buffer A (150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 10mM Tris, pH 7.8) The cells were scraped off with a rubber policeman and transferred to a centrifuge tube. The tube was kept on ice for 10 min and then centrifuged for 3 min at 1000 x g. The supernatant was transferred to a new tube. The pellet was resuspended in 1ml of ice-cold buffer A and again centrifuged for 3 min at 1000 x g. The supernatant was combined with the first. To the combined supernatants, 2 ml of 7 M urea, 350 mM NaCl, 10 mM EDTA, 10 mM Tris pH 7.9, 1% SDS was added. The obtained mixture was extracted with 4 ml of phenol-chloroform (1:1) and the resulting aqueous phase treated with 3 volumes of EtOH. The precipitated RNA was centrifuged and suspended in 100 µl of PBS.

b) Synthesis of cDNA

A series of overlapping cDNA clones from the CDV genome was obtained as outlined below. The procedure is described for generating clones containing the entire N, F and H gene sequences. The M, P and L genes may be isolated in the same manner using specific primers for these genes.

#### c) First strand cDNA

Primers used for first strand cDNA synthesis were selected on the basis of the published sequence of the OP-CDV vaccine strain (Sidhu et al., 1993). They are located in regions which are highly conserved in Morbilliviruses. The 10 primers used and their sequence identification numbers SEQ ID NO 1 to 10 are given hereinafter.

Reaction mixtures for cDNA synthesis contained: 24.5  $\mu$ l H<sub>2</sub>O, 10  $\mu$ l 5X AMV reverse transcription buffer, 1  $\mu$ l of a 75  $\mu$ M dNTP solution, 2.5  $\mu$ l of a 40  $\mu$ M primer solution, 1  $\mu$ l RNase inhibitor, 1  $\mu$ l AMV reverse transcriptase (5 units/ $\mu$ l), 10  $\mu$ l of the above obtained RNA/PBS solution. Samples were incubated for 2 h at 42°C and then heated at 75°C for 10 min.

#### e) Synthesis of double stranded cDNA

Double stranded cDNA was synthesized using polymerase chain reaction (PCR). Reaction mixtures for amplification of a specific region of the CDV genome contained both the 3' and 5' primers (see SEQ ID NOs). Synthesis was performed in a volume of 100  $\mu$ l and contained the following: 77.4  $\mu$ l H<sub>2</sub>O, 10  $\mu$ l 10X Taq buffer, 1.1  $\mu$ l of a solution containing all 4 dNTPs at 20  $\mu$ M each, 0.5  $\mu$ l of a 40  $\mu$ M primer solution, 1  $\mu$ l of Taq polymerase (0.5 units/ $\mu$ l) and 10  $\mu$ l of first strand cDNA, heated to 75°C for 10 min and then cooled on ice. PCR reactions were performed for 30 cycles under standard conditions.

#### f) Cloning of cDNA

PCR amplified cDNA was cloned into the PCR II vector (Invitrogen) using standard conditions (Sambrook et al., 1989).

5 g) Assembly of contiguous genes

The procedure described above for producing cDNA clones resulted in the isolation of the complete N gene.

10 For the F and H genes, a series of overlapping clones was obtained. To assemble these genes into contiguous DNA segments, recombinant PCR (Ho et al., 1989) was used.

Example 2: Preparation of the N Gene

15 Appropriate 5' and 3' ends for insertion of the N gene into expression plasmids were generated by PCR. The following primers were used:

N1, SEQ ID NO 11: 5' GGG GTA CCT CAG GGT TCA  
GAC CTA CCA 3', for generating the 5' end of the gene;  
20 and

N2, SEQ ID NO 12: 5' GCG TCG ACG ACT GAT GTA  
ACA CTG GTC T 3', for generating the 3' end.

This created KpnI and SalI sites at the 5' and 3' ends, respectively. PCR reactions were performed  
25 under standard conditions.

Example 3: Preparation of the F Gene

The primers F1-F4 used in this experiment were designed according to partial sequences of the  
30 A75/17. However, the positions of the underlined nucleotides correspond to the positions of the of the OP-CDV genes according to Barrett et al., 1987. The primers were synthesized with a nucleic acid synthesizer machine.

35 F1, SEQ ID NO 13: 5' CGA CGC GTA GGG TCC AGG  
ACG TAG CA 3', position 1;

F2, SEQ ID NO 14: 5' CAG GTT TAA ATG TCG GAT  
CG 3', position 2033;

F3, SEQ ID NO 15: 5' CGA TCC GAC ATT TAA ACC  
TG 3', position 2014;

5 F4, SEQ ID NO 16: 5' GCGTCG ACA AGA CGT GTG  
ACC AGA GTG 3', position 2095.

The F gene was isolated as 3 overlapping clones. First, the 5' portion of the gene was assembled.  
10 A first cDNA clone containing parts of the M and F genes was cleaved with SacI in the vector DNA and with HindIII at position 687 in the F gene and the fragment of 2035 bp was isolated. A second cDNA clone, containing most of the F gene coding sequences in reverse orientation with re-  
15 spect to the first clone, was also cleaved with HindIII and SacI. The 1405 bp fragment was isolated. Both fragments were ligated into the pBluescript (Stratagene, La Jolla, CA) plasmid cleaved with SacI. To add the 3' end of the F gene, and to generate correct 5' and 3' ends for  
20 cloning into expression plasmids, PCR was used. The 5' portion of the gene was amplified by PCR using primers F1 (5' CGA CGC GTA GGG TCC AGG ACG TAG CA 3') and F2 (5' CAG GTT TAA ATG TCG GAT CG 3') and the DNA fragment was purified by gel electrophoresis on an agarose gel. Similarly,  
25 the 3' portion of the gene was amplified by PCR with primers F3 (5' CGA TCC GAC ATT TAA ACC TG 3') and F4 (5' GCGTCG ACA AGA CGT GTG ACC AGA GTG 3') and purified. Finally, the two parts of the gene were assembled by recombinant PCR using the gel purified 5' and 3' portions of  
30 the gene and primers F1 and F4. This allowed to synthesize the entire F gene as 1 contiguous DNA segment with Mlu I and Sal I sites at the 5' and 3' ends, respectively, for cloning into expression plasmids.

35

#### Example 4: Preparation of the H Gene

The primers H1-H4 used in this experiment were designed according to partial sequences of the



A75/17 genome. However, the positions of the underlined nucleotides correspond to the positions of the OP-CDV genes according to Curran et al., 1991. The primers were synthesized with a nucleic acid synthesizer machine.

5

H1, SEQ ID NO 17: 5' GCG GTA CCA CAA TGC TCT  
CCT ACC AG 3', position 18;

H2, SEQ ID NO 18: 5' CAT ACA CTC CGT CTG AGA  
TAG C 3', position 705;

10

H3, SEQ ID NO 19: 5' GCT ATC TCA GAC GGA GTG  
TAT G 3', position 684;

H4, SEQ ID NO 20: 5' GCG TCG ACT TAA CGG TTA  
CAT GAG AAT CT 3', position 1835:

15

The H gene coding sequences were cloned as 2 overlapping cDNA clones. The gene was assembled by PCR technology. First, the 5' portion of the gene was amplified by PCR using primers H1 (5' GCG GTA CCA CAA TGC TCT CCT ACC AG 3') and H2 (5' CAT ACA CTC CGT CTG AGA TAG C 3') and the resulting DNA fragment was isolated. The 3' portion of the gene was amplified with primers H3 (5' GCT ATC TCA GAC GGA GTG TAT G 3') and H4 (5' GCG TCG ACT TAA CGG TTA CAT GAG AAT CT 3') and the DNA fragment was also isolated. The two portions of the gene were fused in a recombinant PCR reaction containing both DNA fragments and primers H1 and H4. This resulted in the synthesis of a DNA fragment containing the entire H gene coding sequences with a KpnI site at the 5' end and a SalI site at the 3' end for cloning into expression plasmids.

20

#### Example 5: Cloning into eukaryotic expression plasmids

The recombinant PCR products were purified by gel electrophoresis on an agarose gel. The ends were rendered blunt by Klenov polymerase and the fragments were cloned into the EcoRV site of the plasmid pBluescript (Stratagene, La Jolla, CA) and amplified. The inserts

25

were isolated from plasmids containing the F gene by digestion with MluI and SalI and from plasmids harboring the N and H genes by KpnI and SalI.

The fragments were then cloned either into the plasmid pCI (Promega) or into plasmid pCMV-5 (Andersson et al., 1989). The obtained expression plasmids F/CMV5, F/pCI, H/CMV5, HCPI, N/MCV5 and N/pCI were purified according to standard methods and are shown in Figures 2 to 7.

#### Example 6: Preparation of vaccines

Vaccines are prepared by dissolving one or more of the obtained expression plasmids in sterilized PBS of pH 7.4 in a concentration of 1 mg/ml. The vaccine solution may be freshly prepared just before use or filled under sterile conditions in vials of appropriate size.

#### Example 7: Antibody response in mice immunized with N/pCI

The immune response following intramuscular injection of plasmid N/pCI was tested in mice. Two independent experiments were performed. In the first one (Table 1, Experiment No. I), 5 Balb-c mice were injected with plasmid N/pCI purified by the Qiagen procedure (Qiagen Inc, Chatsworth, CA, USA) according to the instructions of the supplier. Five mice were injected with empty vector DNA purified in the same manner. As a further control, 5 animals were injected with PBS alone. In the second experiment (Experiment No. II) 5 mice were injected with plasmid pCI/N purified by cesium chloride gradient centrifugation (Sambrook et al., 1989) and 5 mice with empty vector DNA purified by the same procedure. In both experiments each animal was injected with 100 µg of DNA in PBS at a concentration of 1 mg/ml, receiving 50 µg in each quadriceps muscle per inoculation.

A total of 4 inoculations were performed at biweekly intervals. Two weeks after the last injection the animals were sacrificed and the serum was collected.

Antibody titers were determined by ELISA using serially diluted mouse sera. Maxisorp ELISA plates (Nunc, Roskilde, Denmark) were coated with 50 ng of recombinant N protein per well in carbonate/bicarbonate buffer (15 mM  $\text{Na}_2\text{CO}_3$ , 35 mM  $\text{NaHCO}_3$ , 0.02%  $\text{NaN}_3$ , pH 9.6) at 4°C for 16 hours. After 3 washes with TBS-T (137 mM NaCl, 2.68 mM KCl, 24.7 mM Tris, 0.05 % Tween-20; pH 7.5) the plates were blocked at room temperature for 60 min with PBS-T/LM (PBS containing 0.05% Tween-20 and 2% low fat milk powder. The plates were subsequently washed 3 times with TBS-T before adding 50  $\mu\text{l}$  of the mouse sera diluted in PBS-T/LM. After incubation at 37°C for 60 min. and 3 washes with TBS-T, horseradish peroxidase-labelled goat anti-mouse IgG (Sigma, St. Louis, MO, USA ), diluted 1000-fold in PBS-T/LM was added as the secondary antibody. The plates were incubated at 37°C for 60 min and then washed 3 times with TBS-T. Finally, 50  $\mu\text{l}$  of a solution of 1 mg/ml of 1,2 phenylene-diamine in 0.1 M Na-citrate, pH 5.0, containing 0.001 volumes of 30%  $\text{H}_2\text{O}_2$  was added per well. The reaction was stopped with 50  $\mu\text{l}$  of 4 M  $\text{H}_2\text{SO}_4$  per well, and the optical density was read at a wave length of 490 nm in a Microplate reader 3550 (Bio-Rad Laboratories, Hercules, CA, USA).

The results (Table 1) show that in contrast to control animals, all animals injected with plasmid N/pCI had significant anti-N antibody titers of up to 1:25'600. Intramuscular injection of plasmid N/pCI thus induces a good immune response, demonstrating the usefulness of the proposed vaccine for protecting animals against canine distemper.

Table I: Anti-N antibody titers in mice injected with plasmid N/pCI

Experiment No.	Treatment	Exp.-Mouse No.	Titer
I	PBS	I-1	< 1 : 50
		I-2	< 1 : 50
		I-3	< 1 : 50
		I-4	< 1 : 50
		I-5	< 1 : 50
I	pCI	I-6	1 : 200
		I-7	1 : 200
		I-8	1 : 200
		I-9	1 : 200
		I-10	1 : 200
I	N/pCI	I-11	1 : 800
		I-12	1 : 1600
		I-13	1 : 3200
		I-14	1 : 6400
		I-15	1 : 25600
II	pCI	II-1	< 1 : 50
		II-2	< 1 : 50
		II-3	< 1 : 50
		II-4	< 1 : 50
		II-5	< 1 : 50
II	N/pCI	II-6	1 : 3200
		II-7	1 : 1600
		II-8	1 : 200
		II-9	1 : 12800
		II-10	1 : 3200

Example 8: CTL response in mice immunized  
with N/pCI

Groups of 4 mice were immunized by either 1,  
5 2, or 3 intramuscular injections at 21-day intervals with  
a total of 100 µg of plasmid N/pCI. Control animals were  
injected with empty vector. Twelve days after the first,  
second, or third injection the mice were sacrificed and  
the spleen was removed. Splenocytes were isolated using a  
10 cell strainer and resuspended in DMEM supplemented with  
5% heat-inactivated fetal calf serum, 100 µg/ml penicillin,  
100 U/ml streptomycin, 0.05 mM β-mercaptoethanol, 10  
mM HEPES, and non-essential amino acids. The cells were  
then stimulated by incubation with a synthetic 9 amino  
15 acid peptide (YPALGLHEF) which has been shown to represent  
a CTL epitope in the measles virus N protein  
(Beauverger et al., 1993) and which is conserved in CDV  
strains Onderstepoort and A75/17. The peptide was used at  
a concentration of 10 µM. After 5-7 days the cells were  
20 counted in Trypan blue and adjusted to  $2 \times 10^6$  viable  
cells/ml. The cells were then diluted into microtiter  
plates to yield effector to target cell ratios ranging  
from 100:1 to 0.1:1.

P 815 mastocytoma cells were used as targets  
25 for the CTL assay. Briefly,  $10^6$  cells were incubated for  
1 hour at room temperature with the CTL peptide at a final  
concentration of 1 µM. Control cells were incubated  
in the absence of the peptide. After incubation, the  
cells were centrifuged and resuspended in 100 µl of medium.  
30 Then, 100-150 µCi of  $^{51}\text{Cr}$  was added and the cells  
were incubated for 1 hour at 37°C with occasional shaking.  
The cells were then washed extensively before adding  
 $2 \times 10^3$  target cells per well of effector cells. Target  
and effector cells were incubated 37°C for 4-5 hours. The  
35 plates were then centrifuged and from each well 100 µl of  
medium was removed and the radioactivity was counted in a  
gamma counter. The radioactivity released by control

cells incubated without the CTL peptide was subtracted from the value obtained from cells incubated with the peptide. The resulting value was used to calculate percentage specific lysis.

5 No CTL response was observed after a single immunization (not shown). Importantly, however, after 2 and 3 injections of plasmid N/pCI all mice showed high CTL activity. In contrast, control mice immunized with the empty vector showed very little CTL activity (Fig. 8). Fig. 8 represents CTL assay of mice immunized with plasmid N/pCI or with empty plasmid. Per cent specific lysis was obtained by subtracting the value of non specific lysis of target cells incubated with effector cells in the absence of the CTL peptide. Each curve represents the values obtained with splenocytes from one mouse. Solid line: mice immunized with plasmid N/pCI; broken line: mice immunized with empty vector. The effector (E) to target (T) cell ratio is indicated.

20

#### Example 9: Immunization of dogs with N/pCI

Beagle dogs of 6 weeks of age were used for immunization experiments. Five control animals (Fig. 9, dogs 1-5) received intramuscular injections of a commercially available multivalent vaccine (standard vaccine) containing inactivated canine adenovirus, parainfluenza virus, parvovirus, leptospira and live CDV Onderstepoort strain. Ten dogs (dogs 6-15) were injected into one quadriceps muscle with 100 µg of plasmid N/pCI. Standard vaccine lacking the CDV component was injected into the other quadriceps. A total of 3 injections were performed at 2-week intervals. Before the first, and 2 weeks after each injection (I-III) blood samples were drawn and anti-N antibody levels were determined by ELISA using recombinant CDV N protein as antigen as described for ELISA assays in mice. With standard vaccine, anti N antibody

titers were already elevated with respect to the pre-immune serum after the first vaccination and then reached a plateau. With plasmid N/pCI, in most animals the titers were low after the first and second injection. However, after the third injection, the titers increased and in some animals reached values similar to those obtained with standard vaccine.

The results obtained are visualized in Figure 9. Titers were determined 2 weeks after the first (I), second (II), or third (III) immunization and are represented as the highest serum dilution in which the OD value measured in the ELISA assay was at least twice as high as the value of the corresponding pre-immune serum at the same dilution.

A toxicity test was performed according to the description of the European Pharmacopoeia. Five healthy mice and two healthy guinea pigs were injected with the polynucleotide vaccine as described above. animals were observed for 7 days. None of the animals showed local or systemic reactions.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT:  
(A) NAME: Wittek, Riccardo  
(B) STREET: Ch. de la mésange 1A  
(C) CITY: Vufflens-la-Ville  
10 (E) COUNTRY: Switzerland  
(F) POSTAL CODE (ZIP): 1302
- (i) APPLICANT:  
(A) NAME: Zurbriggen, Andreas  
(B) STREET: Mühlestrasse 158  
15 (C) CITY: Münchenbuchsee  
(E) COUNTRY: Switzerland  
(F) POSTAL CODE (ZIP): 3053
- (ii) TITLE OF INVENTION: Polynucleotide  
20 Vaccine against Canine Distemper
- (iii) NUMBER OF SEQUENCES: 23
- (iv) COMPUTER READABLE FORM:  
25 (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: Patent In Release  
#1.0, Version #1.30 (EPO)  
30
- (v) CURRENT APPLICATION DATA:  
APPLICATION NUMBER:

## 35 (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 nucleotides  
(B) TYPE: deoxyoligonucleotide  
40 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- 45 (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:  
50 (A) ORGANISM:  
(D) DEVELOPMENTAL STAGE:  
(F) TISSUE TYPE:  
(G) CELL TYPE:

(vii) IMMEDIATE SOURCE:  
    (A) LIBRARY:  
    (B) CLONE:  
5      (C) SYNTHETIC: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:  
5': ACC AGA CAA AGT TGG CTA AG  
10

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:  
15      (A) LENGTH: 20 nucleotides  
        (B) TYPE: deoxyoligonucleotide  
        (C) STRANDEDNESS: single  
        (D) TOPOLOGY: linear

20      (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

25      (vi) ORIGINAL SOURCE:  
        (A) ORGANISM:  
        (D) DEVELOPMENTAL STAGE:  
        (F) TISSUE TYPE:  
30      (G) CELL TYPE:

    (vii) IMMEDIATE SOURCE:  
        (A) LIBRARY:  
        (B) CLONE:  
35      (C) SYNTHETIC: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:  
5': ATA ATG AGT TTA GAA GGA GA  
40

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:  
45      (A) LENGTH: 28 nucleotides  
        (B) TYPE: deoxyoligonucleotide  
        (C) STRANDEDNESS: single  
        (D) TOPOLOGY: linear

50      (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:  
5 (A) ORGANISM:  
(D) DEVELOPMENTAL STAGE:  
(F) TISSUE TYPE:  
(G) CELL TYPE:

(vii) IMMEDIATE SOURCE:  
10 (A) LIBRARY:  
(B) CLONE:  
(C) SYNTHETIC: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:  
15 5': CAG TGG ATC CCC AAG TCA GAG TAA TAG A

(2) INFORMATION FOR SEQ ID NO:4:  
20

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 nucleotides  
(B) TYPE: deoxyoligonucleotide  
(C) STRANDEDNESS: single  
25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO  
30

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:  
35 (A) ORGANISM:  
(D) DEVELOPMENTAL STAGE:  
(F) TISSUE TYPE:  
(G) CELL TYPE:

(vii) IMMEDIATE SOURCE:  
40 (A) LIBRARY:  
(B) CLONE:  
(C) SYNTHETIC: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:  
45 5': GGG TCA CGT AAA CTC GGG CC

(2) INFORMATION FOR SEQ ID NO:5:  
50

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 nucleotides  
(B) TYPE: deoxyoligonucleotide

30

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(D) DEVELOPMENTAL STAGE:

(F) TISSUE TYPE:

(G) CELL TYPE:

(vii) IMMEDIATE SOURCE:

(A) LIBRARY:

(B) CLONE:

(C) SYNTHETIC: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

5': GGA CCT CCC GAG CAC GAC AC

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 nucleotides

(B) TYPE: deoxyoligonucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(D) DEVELOPMENTAL STAGE:

(F) TISSUE TYPE:

(G) CELL TYPE:

(vii) IMMEDIATE SOURCE:

(A) LIBRARY:

(B) CLONE:

(C) SYNTHETIC: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

5': TAG GAT TTC GAA GTT CCA GT

## (2) INFORMATION FOR SEQ ID NO:7:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 nucleotides  
(B) TYPE: deoxyoligonucleotide  
(C) STRANDEDNESS: single  
10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

15 (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:  
(A) ORGANISM:  
(D) DEVELOPMENTAL STAGE:  
20 (F) TISSUE TYPE:  
(G) CELL TYPE:

(vii) IMMEDIATE SOURCE:  
(A) LIBRARY:  
25 (B) CLONE:  
(C) SYNTHETIC: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7

30 5': GCA TAC TAA GGT CGA TCC GG

## (2) INFORMATION FOR SEQ ID NO:8:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 nucleotides  
(B) TYPE: deoxyoligonucleotide  
(C) STRANDEDNESS: single  
40 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

45 (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:  
(A) ORGANISM:  
(D) DEVELOPMENTAL STAGE:  
50 (F) TISSUE TYPE:  
(G) CELL TYPE:

(vii) IMMEDIATE SOURCE:



32

- (A) LIBRARY:
- (B) CLONE:
- (C) SYNTHETIC: YES

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8

5': CTG AGG ATC AGA AGT TTT GCC ATA CAC GCC

10 (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 nucleotides
  - (B) TYPE: deoxyoligonucleotide
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA

20 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM:
  - (D) DEVELOPMENTAL STAGE:
  - (F) TISSUE TYPE:
  - (G) CELL TYPE:

- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY:
  - (B) CLONE:
  - (C) SYNTHETIC: YES

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9

35 5': TGC TGA CCG CTA TCT CAG AC

40 (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 nucleotides
  - (B) TYPE: deoxyoligonucleotide
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: DNA

50 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

33

(A) ORGANISM:  
(D) DEVELOPMENTAL STAGE:  
(F) TISSUE TYPE:  
(G) CELL TYPE:

5

(vii) IMMEDIATE SOURCE:  
(A) LIBRARY:  
(B) CLONE:  
(C) SYNTHETIC: YES

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10

5': GGG CTA TCT AGA TGG ACC TC

15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 nucleotides  
(B) TYPE: deoxyoligonucleotide  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA

25

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

30

(vi) ORIGINAL SOURCE:  
(A) ORGANISM:  
(D) DEVELOPMENTAL STAGE:  
(F) TISSUE TYPE:  
(G) CELL TYPE:

35

(vii) IMMEDIATE SOURCE:  
(A) LIBRARY:  
(B) CLONE:  
(C) SYNTHETIC: YES

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11

5' GGG GTA CCT CAG GGT TCA GAC CTA CCA 3'

45

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 nucleotides  
(B) TYPE: deoxyoligonucleotide  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: DNA  
(iii) HYPOTHETICAL: NO  
5 (iv) ANTI-SENSE: NO  
(vi) ORIGINAL SOURCE:  
    (A) ORGANISM:  
    (D) DEVELOPMENTAL STAGE:  
10 (F) TISSUE TYPE:  
    (G) CELL TYPE:  
(vii) IMMEDIATE SOURCE:  
    (A) LIBRARY:  
15 (B) CLONE:  
    (C) SYNTHETIC: YES  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12  
20 5' GCG TCG ACG ACT GAT GTA ACA CTG GTC T 3'  
(2) INFORMATION FOR SEQ ID NO:13:  
25 (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 26 nucleotides  
    (B) TYPE: deoxyligonucleotide  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
30 (ii) MOLECULE TYPE: DNA  
(iii) HYPOTHETICAL: NO  
35 (iv) ANTI-SENSE: NO  
(vi) ORIGINAL SOURCE:  
    (A) ORGANISM:  
    (D) DEVELOPMENTAL STAGE:  
40 (F) TISSUE TYPE:  
    (G) CELL TYPE:  
(vii) IMMEDIATE SOURCE:  
    (A) LIBRARY:  
45 (B) CLONE:  
    (C) SYNTHETIC: YES  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13  
50 5' CGA CGC GTA GGG TCC AGG ACG TAG CA 3'  
(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 20 nucleotides  
    (B) TYPE: deoxyoligonucleotide  
5      (C) STRANDEDNESS: single  
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

10      (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:  
15      (A) ORGANISM:  
        (D) DEVELOPMENTAL STAGE:  
        (F) TISSUE TYPE:  
        (G) CELL TYPE:

20      (vii) IMMEDIATE SOURCE:  
        (A) LIBRARY:  
        (B) CLONE:  
        (C) SYNTHETIC: YES

25      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14

5' CAG GTT TAA ATG TCG GAT CG 3'

30      (2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 20 nucleotides  
    (B) TYPE: deoxyoligonucleotide  
35      (C) STRANDEDNESS: single  
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

40      (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:  
45      (A) ORGANISM:  
        (D) DEVELOPMENTAL STAGE:  
        (F) TISSUE TYPE:  
        (G) CELL TYPE:

50      (vii) IMMEDIATE SOURCE:  
        (A) LIBRARY:  
        (B) CLONE:  
        (C) SYNTHETIC: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15

5' CGA TCC GAC ATT TAA ACC TG 3'

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 nucleotides
- (B) TYPE: deoxyoligonucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:
- (D) DEVELOPMENTAL STAGE:
- (F) TISSUE TYPE:
- (G) CELL TYPE:

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY:
- (B) CLONE:
- (C) SYNTHETIC: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16

5' GCG TCG ACA AGA CGT GTG ACC AGA GTG 3'

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 nucleotides
- (B) TYPE: deoxyoligonucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:
- (D) DEVELOPMENTAL STAGE:

37

(F) TISSUE TYPE:

(G) CELL TYPE:

(vii) IMMEDIATE SOURCE:

(A) LIBRARY:

(B) CLONE:

(C) SYNTHETIC: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17

5' GCG GTA CCA CAA TGC TCT CCT ACC AG 3'

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 nucleotides

(B) TYPE: deoxyoligonucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(D) DEVELOPMENTAL STAGE:

(F) TISSUE TYPE:

(G) CELL TYPE:

(vii) IMMEDIATE SOURCE:

(A) LIBRARY:

(B) CLONE:

(C) SYNTHETIC: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18

5' CAT ACA CTC CGT CTG AGA TAG C 3'

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 nucleotides

(B) TYPE: deoxyoligonucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(D) DEVELOPMENTAL STAGE:

(F) TISSUE TYPE:

(G) CELL TYPE:

(vii) IMMEDIATE SOURCE:

(A) LIBRARY:

(B) CLONE:

(C) SYNTHETIC: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19

5' GCT ATC TCA GAC GGA GTG TAT G 3'

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 nucleotides

(B) TYPE: deoxyoligonucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(D) DEVELOPMENTAL STAGE:

(F) TISSUE TYPE:

(G) CELL TYPE:

(vii) IMMEDIATE SOURCE:

(A) LIBRARY:

(B) CLONE:

(C) SYNTHETIC: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20

5' GCG TCG ACT TAA CGG TTA CAT GAG AAT CT 3'

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1678 nucleotides  
 (B) TYPE: cDNA  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: CANINE DISTEMPER VI-RUS, STRAIN CDV A75/17

(D) DEVELOPMENTAL STAGE:

(F) TISSUE TYPE:

(G) CELL TYPE:

(vii) IMMEDIATE SOURCE:

(A) LIBRARY:

(B) CLONE: N/CMV5 OR N/PCI

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21

```

25  AGGGTCAATG ATCCTACCTT AGAGAACAAG GTCAGGGGTC AGACCTACCA ATATGGCTAG      60
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    AAGCATTGTT ACAAGATCTC GACTATTGGA TAGACTTGTT AGATTGGTCG GTGATCCGGA      240
    AATCAACGGG CCTAAATTAA CTGGGATTTT AATCAGTATC CTCTCCTTGT TCGTGGGAATC      300
    CCCTGGACAG TTGATCCAGA GGCATCATA CGACCCGTAT ATAAGCATCA AGTTAGTAGA      360
    GGTAATCCCA AGCATCAACT CTGTTTGGCG TCTTACATT GCATCCAGAG GAGCAAGTTT      420
    GGATTCTGAG GCAGATGAGT TCTTCAAAAT TGTAGACGAA GGGTCGAAAG CTCGAAGGACA      480
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    CAATATATTG CTAGCTTCCA TCTTGGCCCA AATTGGATC CTGCTAGCTA AAGCGGTGAC      600
    TGCTCCTGAT ACTGCAGCCG ACTCGGAGAT GAGAAGGTGG ATTAAGTATA CCCACAGAG      660
    ACGTGTGGTC GGGGAATTCA GAATGAACAA AATATGGCCT GATATTGTGA GAACACAGAT      720
    TGCTGAGGAC TTATCTTTGA GCGGTTTCAT GGTGGCACTC ATCTTGGATA TCAACGATC      780
    CCCAGGGAAC AAGCCTAGAA TTGCTGAAAT GATTGTGAT ATAGATAACT ACATTGTGGA      840
    AGCTGGATTA GCTAGTTTCA TCTTAACAT CAAATTGGC ATTGAACTA TGTATCCGGC      900
    TCTTGGCTTG CATGAGTTT CCGGAGAGTT AACAACATAT GAATCCCTTA TGTATCTATA      960
    TCAACAGATG GGTGAACAG CACCGTACAT GGTATTCTG GAAATTCTG TTCAGAACAA      1020
    ATTAGTGCA GATCCTACC CATGTCTCTG GAGTTATGCT ATGGGAGTTG GTGTTGAATC      1080
    TGAAAACTCC ATGGGAGGGT TAAATTTCGG TAGATCTTAC TTGTATCCAG CTATTCTCAG      1140
    GCTCGGGCAA GAAATGGTTA GAAGATCTGC CGGCAAGATA AGCTCTGCAC TTGCGCCGGA      1200
    GCTTGGCATC ACCAAGGAAG AGGCTCAACT AGTGTAGAA ATAGCATCCA AGACAACGGA      1260
    GGACCGGACG ATTCGCGCTG CTGCTCCCAA GCAATCCAA ATCATTCTT TGCATCTAGA      1320
    AAGATCCGAA GTCATAACT AACAACCCCC ACCATCAAC AAGAGGTCGG AAAACCAAGG      1380
    AGGAGACAAA TACCCCATCC ACTTCAGTGA TGACCCCAA ACTATTCAAG ATGATGGAAA      1440
    CAACAGCTCC GAATGGAGTG AATCAGCTA TGATACCCAA AGAATGCTTA CTAAGATGCT      1500
    CGACGATGAC CGGAATCGA TGGAGCAAT CGCCAGATG AGAATGCTTA CTAAGATGCT      1560
    CAGTCAACTG GGGACCACTG AAGAGAGTTC TCCTGTCTAT AATGATAGAG AGCTACTCAA      1620
    TTAATATATC AAGACCAGTG TTACATCAGT CAACGATTCT CTTCTAAAC TCATTATA      1678
  
```

55 (2) INFORMATION FOR SEQUENCE ID NO: 22

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2198

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear



(ii) MOLECULAR TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "deoxynucleotide"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: Canine distemper virus  
(B) STRAIN: A75/17

(viii) POSITION IN GENOME:  
(B) MAP POSITION: F gene position is 5' end of F mRNA  
(C) UNITS: bp

(ix) FEATURE:  
(A) NAME/KEY: mRNA  
(B) LOCATION: complement (1....2198)

25 AGGGTCCAGG ACGTAGCAAG CCTACAGGCC AACCAAGTCC ACCAACTCTA GGCCGGGCGAG 60  
GAACCCCCAC GAACAGACAA GCCCCATGCA CAACAAAATC CCCAAAAGGT CCAACACCCCG 120  
AAAACACACC CAACAGAGACC TCCCCCCACA ACACAGCACC AAATCCGCCG AGACCAAGAC 180  
CTCCCAAGCA CGACACAGCA CAACATCGGC TCGGCGATCC ACGCACCATG GTCTCTTAAC 240  
ATCGGACAGG CCCATCCACT ACATCATGAA CAGGATCAGG TCCGTCAAGC AAGCCAGCCA 300  
30 CAGATCCGGT AACATCCCGG CTCACGGAGA CCATGAGGGC ACCATCCATC ACACACCAGG 360  
GAGTGTCTCC CAAGGAGCGG GATCCCGGCT CAAAAGGCGG CAATCCAATG CAACCAACTC 420  
AGGCTCTCAG TGCACCTGGT TAGTCCTATG GTGCATTGGA ATAGCCAGTC TCCTTCTTTG 480  
TTCTAAGGCT CAGATACATT GGAATAATT GTCAACTATT GGGATTATCG GGACTGACAG 540  
TGTCATTAT AAGATCATGA CTAGACCCAG TCACCAGTAC TTGGTCATTA AACTAATGCC 600  
35 TAATGTTTCA CTTATAGATA ATTGTACCAA AGCAGAAATTA GGTGAGTATG AGAAATTAAT 660  
AAATTCAGTC CTCGAGCCAA TCAATCAAGC TTTGACTCTA ATGACCAAGA ATGTGAAGCC 720  
CCTACAGTCA GTAGGGGTGAG GTAGGAGACA AAGGCGTTTT GCAGGAGTGG TGCTTGACAGG 780  
TGCAGCTTTA GGAGTAGCCA CAGCTGCACA AATCACTGCA GGGATAGCTT TACATCAATC 840  
CAACCTCAAT GCTCAAGCAA TCCAACTCTT GAGAACTAGC CTTGAACAGT CCAACAAGGC 900  
40 TATAGAAGAA ATTAGGGAGG CAACCCAGGA AACCGTCATT GCCGTTCAGG GAGTTCAGGA 960  
TTACGTCAT AATGAATCG TCCCTGCTAT GCAACATATG TCGTGTGAAT TAGTTGGGCA 1020  
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AGAAATTCAT AAGATACTTG AGAAGTTGGG ATATTCTGGA AATGATATGA TTGCAATTTT 1200  
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AGGCATTGGT AGCCAGAATC CCGTATACCC CATGAGCCCG CTTCACAAE AATCAGATTAG 1500  
50 GGGTGACACT TCATCTCTCG CTCGGACCTT GGTGCTCGGG ACGATGGGCA ACAAGTTTAT 1560  
TCTGTCAAAA GGTAAATACG GTCTGTATAA ATTGCTGACA TTTATTGCCCT CCGGTACCTG 1620  
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CCCACTGGTT GAGATAGATG GTGTAATAT CCAGGTTGGA GGGAGGCAAT ACCCTGATAT 1740  
GGATTACGAA AGCAAAAGTT CCTTAGGCCG TGCTATATCA CTTGAGAGGT TAGATGTAGG 1800  
55 TACAAATTTA GGGAGCGCCC TTAAGAAACT GGATGATGCT AAGGTACTGA TAGACTCCCT 1860  
TAACAGATTC CTTGAGACCG TTAGGCGCTC TTCTTTAAT TTTGGCAGTC TTCTCAGCGT 1920  
TCCCATATTA ATATGTACAG CCCTGGCTTT GTTGTGCTG ATTACTGCT GTAAAGAGCG 1980  
CTACCAACAG ACCTCAAGC AGAATGCTAA GGTGATCCG ACATTTTAAG CTGATTTGAC 2040  
TGGAACTTGG AATCTCTATG TAAGATCACT CTAAGCACT CTGGTCACAC GTCTTACCCG 2100  
60 ATGTGACGG TTGAATCTTA TAAATCCCCC CCAATTTTCT TCAAAAGCTA TCAAACTACA 2160  
ACAAATAGTG GAGAGGACTG ACTACGATTA TCGTAATT 2198

## (2) INFORMATION FOR SEQUENCE ID NO: 23

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1969  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULAR TYPE: other nucleic acid

- (A) DESCRIPTION: /desc =

"deoxynucleotide"

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (v) FRAGMENT TYPE: internal

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Canine distemper virus  
 (B) STRAIN: A75/17

## (viii) POSITION IN GENOME:

- (B) MAP POSITION: H gene position is

25 5' end of H mRNA

- (C) UNITS: bp

## (ix) FEATURE:

- (A) NAME/KEY: mRNA  
 (B) LOCATION: complement (1....1969)

```

AGGGCTCAGG TAGTCCAACA ATGCTCTCCT ACCAGGACAA GGTGAGTGCC TTCTATAAGG      60
ATAATGCAAG AGCTAATICA TCCAAGCTAT CCTTAGTGAC AGAAGAGCAA GGGGGCAGGA      120
GACCAACCCTA TTTGCTGTTT GTCCCTTCTCA TCCTACTGGT TGGATCATG GCCCTGCTTG      180
CTATCACTGG AGTTCGATTT CACCAAGTAT CAACTAGCAA TATGGAATTT AGCAGATTGC      240
TGAAGAGGGA TATGGAGAAA TCAGAGGCCG TACATCACC AATCATAGAT GTCTTGACAC      300
CGCTCTTCAA AATTATTGGA GATGAGATTG GGTACGGTT GCCACAAAA CTAAACGAGA      360
TCAACCAATT TATCTTCAA AAGACAACT TCTTCAATCC GAACAGGGAG TTCGACTTCC      420
GCGATCTCCA CTGGTGCAAT AACCCACCTA GTAAGATCAA AGTGAATTT ACTAATTACT      480
GCGATACAAT TGGGATCAGA AAATCTATTG CATCGGCAGC AAATCCTATC CTTTATCAG      540
CACTCTCCGG AGGCAGAGGT GACATATTCC CACCATACAG ATGCAGTGGG GCTACTACTT      600
CAGTAGGCAG AGTTTTCCCC CTATCAGTAT CATTGTCCAT GTCTTTGATC TCAAGAACAT      660
CAGAGATAAT CAATATGCTA ACCGCTATCT CAGACGGAGT GTATGGTAAA ACTTATTTCG      720

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	TAGTTCATGA	TTATATTGAA	GGGGGGTTG	ACACGCCAAA	GATTCGAGTC	TTTGAGATAG	780
	GGTTCATCAA	ACGGTGGCTG	AATGACATGC	CATTACTCCA	GACAACCAAC	TATATGGTCC	840
	TCCTGGAGAA	TTCCAAAGCC	AAGGTATGTA	CTATACGGGT	GGCGGAGTTG	ACACTGGCTT	900
	CCTTGTGTGT	AGATGAGAGC	ACCGTATTGT	TATATCATGA	CAGCGATGGT	TCACAAGATG	960
5	GTATTCTAGT	GGTGACCGTG	GGAAATATTG	GGGCAACACC	TATGGATCAA	GTGGAAGAGG	1020
	TGATACCTGT	TGCTCACCCA	TCAGTAGAAA	AAATACATAT	AACAAATCAC	CGTGGGTTCA	1080
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	CACTGGGAACC	CTTTGGAGGA	GGACAGTTCC	CATCTTATGG	GCGGTTGACA	TTACCTCTAG	1260
10	ATCCAGCAT	TGACCTTCAA	CTTAACATCT	CGTTTACATA	CGGTCCGGCT	ATACTGAATG	1320
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	ATTTAGTCCG	TATAAGATTG	TCATGTAATC	GTTCAAAACC	TTGACAGTAT	GATGATACAC	1860
20	ATTTCAATTG	GACTTAGGTA	TGATGACTGT	GGTGAGAAAT	TCCTTACCBA	CGATTGAATT	1920
	AAACCATCTC	CAGCATTATA	AAAAAACTAA	GGATCCAGGA	TCCTTTTAG		1969

## CLAIMS

1. A nucleic acid construct comprising one or  
5 more canine distemper virus gene, wherein said nucleic  
acid construct is capable of inducing the expression of  
an antigenic canine distemper virus gene product which  
induces a canine distemper virus specific immune response  
upon introduction of said nucleic acid construct into  
10 animal tissue *in vivo* and resultant uptake of the nucleic  
acid construct by the cells which express the encoded ca-  
nine distemper virus gene.
2. A nucleic acid construct according to  
15 Claim 1, wherein the canine distemper virus gene encodes  
the nucleocapsid (N) protein, the phosphoprotein (P), the  
matrix (M) protein, the fusion (F) protein, the hemagglu-  
tinin (H) protein, or the large (L) protein.
- 20 3. A nucleic acid construct according to  
Claim 1 or 2, wherein the canine distemper virus gene en-  
codes the nucleocapsid (N) protein, the fusion (F) pro-  
tein, or the hemagglutinin (H) protein.
- 25 4. A DNA construct according to anyone of  
Claims 1 to 3, which is the plasmid N/CMV5 or N/pCI,  
which encode the nucleocapsid (N) protein, the plasmid  
H/CMV5 or H/pCI, which encode the hemagglutinin (H) pro-  
tein, or the plasmid F/CMV5 or F/pCI which encode the fu-  
30 sion (F) protein of canine distemper virus strain A75/17.
5. A polynucleotide vaccine comprising an ef-  
fective amount of a DNA or RNA construct according to  
anyone of Claims 1 to 4 and a physiologically acceptable  
35 carrier.

6. A polynucleotide vaccine according to Claim 5 which induces neutralizing antibodies against canine distemper virus, canine distemper virus specific cytotoxic lymphocytes, or protective immune responses upon introduction of said vaccine into animal tissue *in vivo*, wherein the animal is a mammal, carnivore, in particular a dog, or a human.

7. A polynucleotide vaccine according to Claim 5 or 6 comprising one or more of the plasmids selected from N/CMV5 or N/pCI, which encode the nucleocapsid (N) protein, H/CMV5 or H/pCI, which encode the hemagglutinin (H) protein, or F/CMV5 or F/pCI which encode the fusion (F) protein of canine distemper virus strain A75/17 and a vaccine carrier.

8. A polynucleotide vaccine according to any one of Claims 5 to 7 additionally comprising further components to form a multivalent vaccine.

9. A method for protecting an animal susceptible to canine distemper infection against disease by canine distemper virus which comprises immunization of said animal with a prophylactically effective amount of a polynucleotide vaccine of anyone of claims 5 to 8.

10. A method according to Claim 9, wherein the animal is a mammal, such as a carnivore, in particular a dog.

11. A method according to Claim 9 or 10, wherein at least one polynucleotide is administered directly into the animal tissue *in vivo*.

12. A method according to Claims anyone of 9 to 11, wherein the polynucleotide is administered either in naked form in a physiologically acceptable solution,

or contained in a liposome, or in a mixture with an adjuvant or a transfection facilitating agent.

13. A method for using a canine distemper virus gene to induce an immune response in vivo which comprises:

- a) isolating the gene
- b) linking the gene to regulatory sequences such that the gene is operatively linked to control sequences which, when introduced into a living tissue, direct the transcription initiation and subsequent translation of the gene, and
- c) introducing the gene into a living tissue of an animal susceptible to canine distemper.

15

14. A method according to Claim 13, which comprises multiple introduction of the canine distemper virus gene for boosting the immune response.

15. A method according to Claim 13 or 14, wherein the canine distemper virus gene encodes the nucleocapsid (N) protein, the hemagglutinin (H) protein, or the fusion (F) protein of canine distemper virus strain A75/17.

25

16. A method according to anyone of Claims 13 to 15, wherein the canine distemper virus gene product for immunization is selected from the plasmids F/CMV5 or F/pCI, which encode the fusion (F) protein, H/CMV5 or H/pCI, which encode the hemagglutinin (H) protein, or N/CMV5 or N/pCI which encode the nucleocapsid protein of canine distemper virus strain A75/17.

17. A composition of nucleic acid constructs encoding canine distemper genes from more than one canine distemper strain.

18. The use of an isolated canine distemper virus gene operatively linked to one or more control sequences for the preparation of a vaccine for use in immunization against disease by canine distemper virus.

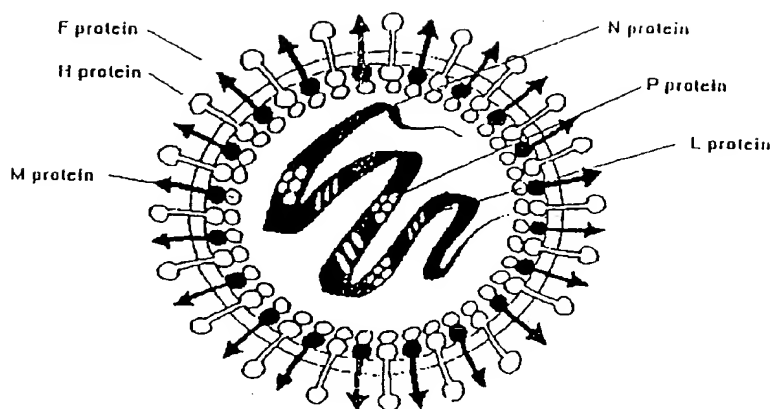


Fig. 1



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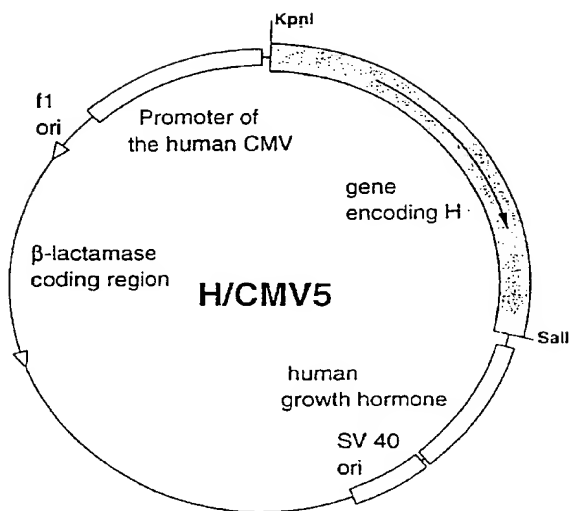


Fig. 2

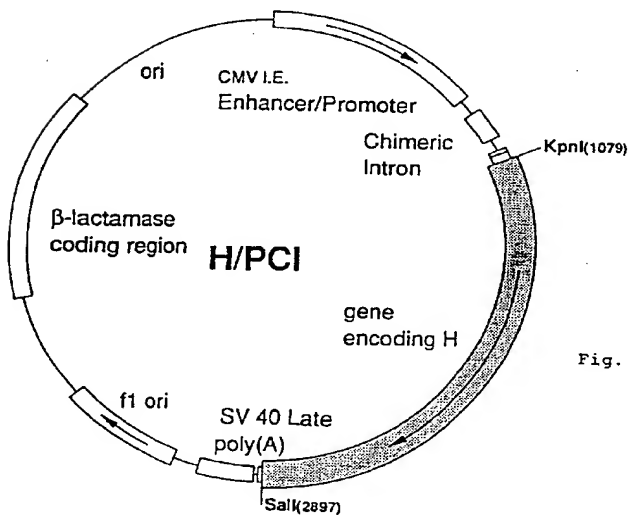


Fig. 3

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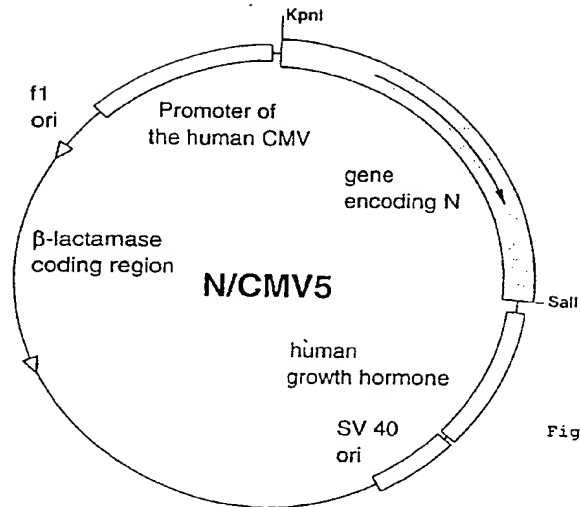


Fig. 4

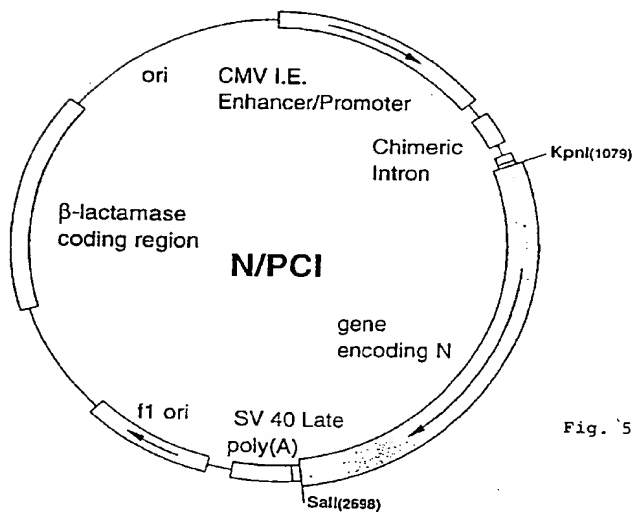


Fig. 5

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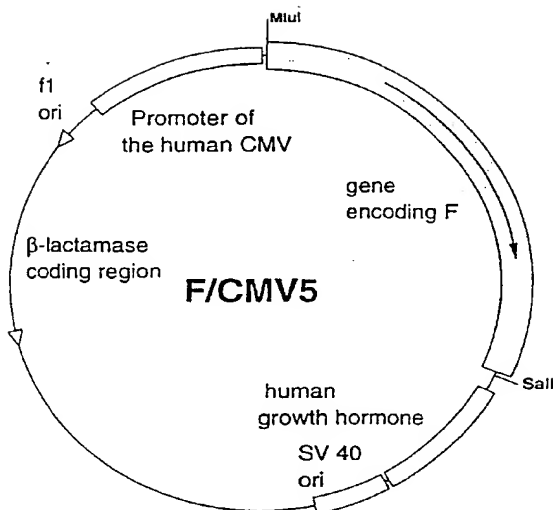


Fig. 6

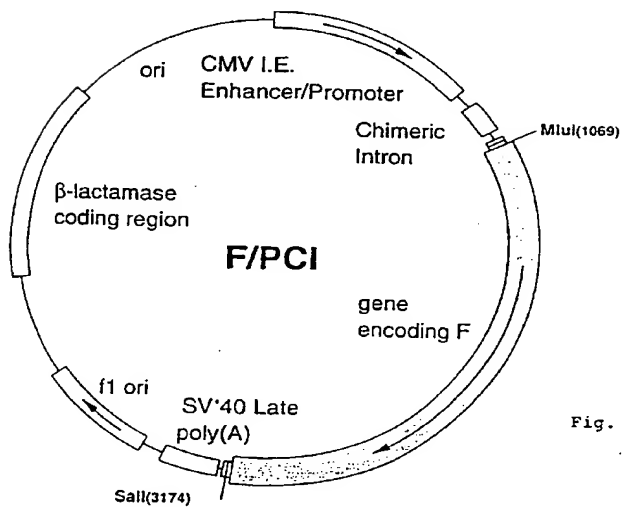


Fig. 7

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## CTL assays

after 2nd immunization

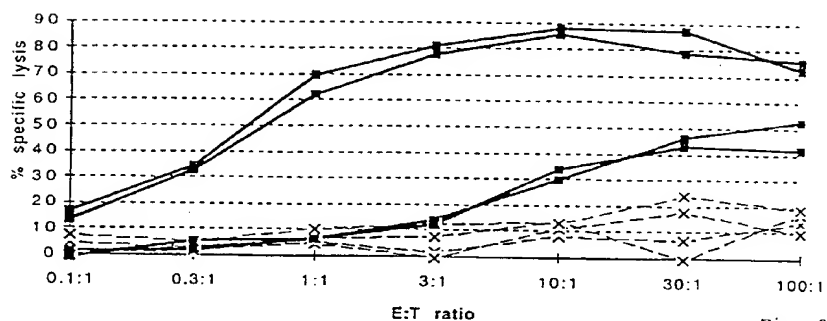
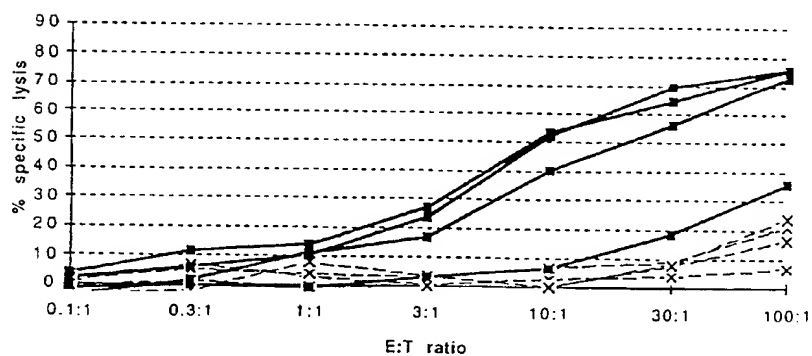


Fig. 8

after 3rd immunization



x-----x mice immunized with empty vector

■-----■ mice immunized with plasmid N/pCI

Fig. 9

SUBSTITUTE SHEET (RULE 26)

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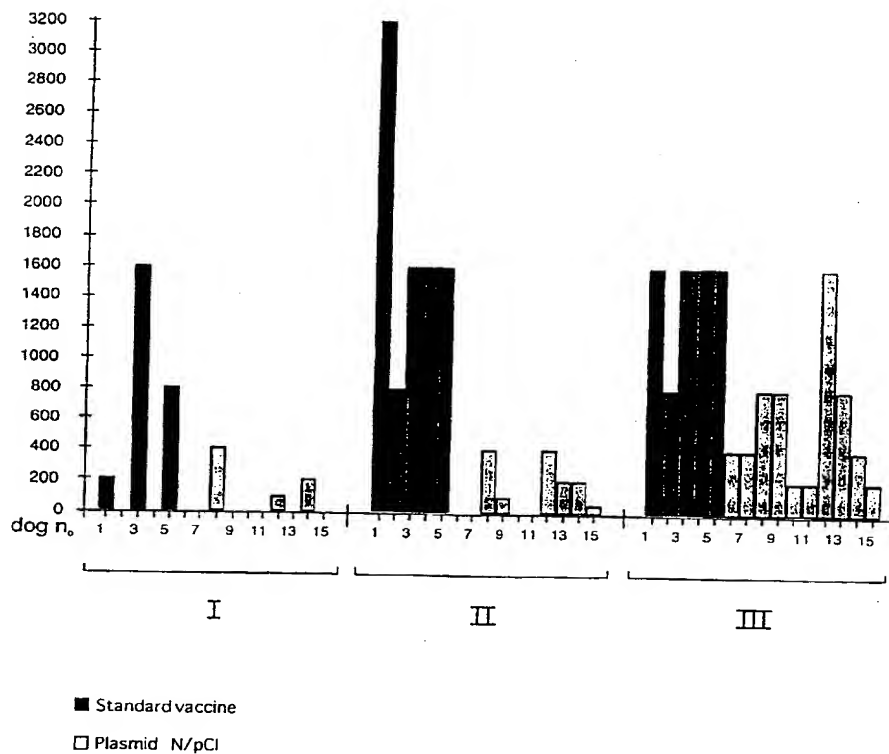


Fig. 10

SUBSTITUTE SHEET (RULE 26)

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/IB 97/00444

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/45 A61K31/70 A61K39/175

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 27780 A (VIROGENETICS CORPORATION) 19 October 1995 see page 108, line 19 - page 121, line 16	1-18
A	VETERINARY MICROBIOLOGY, vol. 44, no. 2-4, 1995, pages 211-217, XP000601698 M.STETTLER AND A.ZURBRIGGEN: "Nucleotide and deduced amino acid sequences of the nucleocapsidprotein of the virulent A75/17-CDV strain of canine distemper virus" see the whole document --- -/--	1-18

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \* "A" document defining the general state of the art which is not considered to be of particular relevance
- \* "E" earlier document but published on or after the international filing date
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- \* "O" document referring to an oral disclosure, use, exhibition or other means
- \* "P" document published prior to the international filing date but later than the priority date claimed

\* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\* "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\* "A" document member of the same patent family

Date of the actual completion of the international search

5 August 1997

Date of mailing of the international search report

14.08.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

Int. Appl. No.  
PCT/IB 97/00444

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCHWEIZER ARCHIV FÜR TIERHEILKUNDE, vol. 138, no. 2, February 1996, pages 99-103, XP000601356 H.U.GRABER ET AL.: "RT-PCR:Ein Hilfsmittel zur Herstellung von Klonen für die Staupeforschung" see the whole document ---	1-18
A	AMERICAN JOURNAL OF VETERINARY RESEARCH, vol. 54, no. 9, September 1993, pages 1457-1461, XP000601924 A.ZURBRIGGEN ET AL.: "In situ hybridization of virulent canine distemper virus in brain tissue, using digoxigenin-labeled probes" see the whole document ---	1-18
A	DNA AND CELL BIOLOGY, vol. 12, no. 9, November 1993, pages 777-783, XP000565708 D.L.MONTGOMERY ET AL.: "Heterologous and homologous protection against influenza A by DNA vaccination: Optimizing of DNA vectors" see the whole document -----	1-16

Form PCT/ISA/218 (continuation of second sheet) (July 1992)

page 2 of 2

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 97/00444

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim(s) 9-16  
is(are) directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No  
PCT/IB 97/00444

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9527780 A	19-10-95	AU 2283395 A	30-10-95
		CA 2187207 A	19-10-95
		EP 0759072 A	26-02-97
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Form PCT/ISA/210 (patent family annex) (July 1992)